

(19) World Intellectual Property Organization International Bureau



23 MAR 2005 528848



(43) International Publication Date  
8 April 2004 (08.04.2004)

PCT

(10) International Publication Number  
WO 2004/029275 A1

(51) International Patent Classification<sup>7</sup>: C12P 23/00 (74) Agents: KELLER, Günter et al.; Lederer & Keller, Prinzregentenstrasse 16, 80538 München (DE).

(21) International Application Number:

PCT/EP2003/010574

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(22) International Filing Date:

23 September 2003 (23.09.2003)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

02021599.2 27 September 2002 (27.09.2002) EP

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/029275 A1

(54) Title: PRODUCTION OF ZEAXANTHIN BY PHAFFIA

(57) Abstract: Disclosed is a process for producing zeaxanthin and  $\beta$ -cryptoxanthin which comprises cultivating a recombinant microorganism which is expressing a  $\beta$ -carotene hydroxylase gene and belonging to the genus *Xanthophyllomyces* (Phaffia) in an aqueous nutrient medium under aerobic conditions, and isolating the resulted carotenoids from the cells of said recombinant microorganism or from the cultured broth.

Production of zeaxanthin by *Phaffia*

The present invention relates to production of xanthophyll carotenoids, especially zeaxanthin and  $\beta$ -cryptoxanthin by microorganism belonging to genus *Phaffia*.

More particularly, the present invention provides a process for producing xanthophyll carotenoids, especially zeaxanthin and  $\beta$ -cryptoxanthin by genetically modified recombinant microorganism belonging to genus *Phaffia*.

By using methods of the present invention, it becomes possible to produce the useful xanthophyll carotenoids other than astaxanthin such as zeaxanthin and  $\beta$ -cryptoxanthin as a major xanthophyll carotenoid in the recombinant microorganism belonging to the genus *Phaffia*.

Any strain which is able to produce  $\beta$ -carotene can be a suitable host strain. When the selected host strain is a normal *Phaffia* strain which is capable of producing astaxanthin from  $\beta$ -carotene, production of mixture of astaxanthin and other xanthophyll carotenoids can be achieved after introduction and expression of a gene coding for  $\beta$ -carotene hydroxylase. On the other hand, when a strain which can not produce astaxanthin and is accumulating  $\beta$ -carotene is selected as the host strain, it is expected to produce maximum level of xanthophyll carotenoids such as zeaxanthin and  $\beta$ -cryptoxanthin, without accumulation of astaxanthin. Such a host strain which accumulates  $\beta$ -carotene can be obtained by mutagenesis of a strain of *P. rhodozyma* accumulating astaxanthin.

Alternatively, such a host strain accumulating  $\beta$ -carotene can also be obtained by deactivating astaxanthin synthase which is an enzyme involved in the biosynthesis of

astaxanthin from  $\beta$ -carotene and is disclosed in US 6,365,386. Disruption of the gene of astaxanthin synthase will be one of the most convenient ways to deactivate the enzyme.

Furthermore, such a host strain accumulating  $\beta$ -carotene can also be obtained from public type culture collections. E.g., *P. rhodozyma* ATCC96815 accumulating  $\beta$ -carotene can be 5 purchased from American Type Culture Collection (P.O.Box 1549, Manassas, VA 20108, USA). Isolating a new  $\beta$ -carotene accumulating strain of *P. rhodozyma*, which may be a derivative or a spontaneous mutant of astaxanthin producing strain, from nature will be another approach to prepare the host strain of the present invention.

An aspect of the present invention is a process for producing zeaxanthin and  $\beta$ -cryptoxanthin 10 which comprises cultivating a recombinant *Phaffia* strain which is expressing  $\beta$ -carotene hydroxylase.

Said  $\beta$ -carotene hydroxylase catalyzes hydroxylation of  $\beta$ -carotene at positions 3 and 3' on the  $\beta$ -ionone ring to produce the xanthophyll, zeaxanthin via  $\beta$ -cryptoxanthin. Genes encoding this enzyme have been isolated from several species, e.g., *crtZ* from *Flavobacterium* 15 species (US 6,124,113), *crtZ* from *Erwinia uredovora*, *crtZ* from *Erwinia herbicola*, *crtZs* from marine bacteria, *Agrobacterium aurantiacum* and *Alcaligenes* sp., and *Paracoccus marcusii* (GenBank accession No. Y15112, 1997), and *Paracoccus carotinifaciens* sp.nov.  $\beta$ -carotene hydroxylase genes can also be obtained from plant species.

In the present invention, any gene encoding a protein having the  $\beta$ -carotene hydroxylase 20 activity can be used.

Preferably, said  $\beta$ -carotene hydroxylase gene can be obtained from a microorganism selected from microorganisms of the genera *Flavobacterium*, *Erwinia*, *Agrobacterium*, *Alcaligenes*, and *Paracoccus* having the  $\beta$ -carotene hydroxylase gene.

More preferably, said  $\beta$ -carotene hydroxylase gene can be obtained from *Flavobacterium* 25 sp. R1534 WT (ATCC21588) (GenBank accession No. U62808), *E. uredovora* ATCC19321 (GenBank accession No. D90087), *E. herbicola* ATCC39368 (GenBank accession No. M87280), *Agrobacterium aurantiacum* (GenBank accession No. D58420), *Alcaligenes* PC-1 (GenBank accession No. D58422), *Paracoccus marcusii* MH1 (GenBank accession No. Y15112), or the gram-negative bacteria E-396 (FERM BP-4283) (JP-A Hei 10-155497) 30 having the  $\beta$ -carotene hydroxylase gene.

Still more preferably, said  $\beta$ -carotene hydroxylase gene can be obtained from *Flavobacterium* sp. R1534 WT (ATCC21588) (GenBank accession No. U62808), or it can also be obtained as a DNA sequence which is substantially homologous thereto.

The expression "a DNA sequence which is substantially homologous" refers with respect to

5 the DNA sequence encoding the  $\beta$ -carotene hydroxylase to a DNA sequence which encodes an amino acid sequence which shows more than 60%, preferably more than 70%, more preferably more than 80%, and most preferably more than 90% identical amino acids when compared to the amino acid sequence of crtZ of *Flavobacterium* sp. R1534 WT (ATCC21588) and is the amino acid sequence of a polypeptide which shows the same type

10 of enzymatic activity as the enzyme encoded by crtZ of *Flavobacterium* sp. R1534 WT(ATCC21588).

By using the  $\beta$ -carotene hydroxylase gene, it is possible to render a microorganism belonging to the genus *Phaffia* an ability to produce zeaxanthin and  $\beta$ -cryptoxanthin. The recombinant microorganism of *Phaffia* expressing a  $\beta$ -carotene hydroxylase gene can be prepared by the well-known recombinant technology.

The techniques used to isolate or clone a DNA encoding  $\beta$ -carotene hydroxylase of the present invention are known in the art and include isolation from genomic DNA. The cloning of the DNA sequence of the present invention from such genomic DNA can be effected by using the polymerase chain reaction (hereinafter referred to as PCR).

20 The isolated or cloned DNA encoding  $\beta$ -carotene hydroxylase can be preferably utilized after cloning on a suitable expression vector for expression of the enzyme in the host microorganism *Phaffia*.

"Expression vector" includes vectors which are capable of expressing DNA sequences contained therein where such sequences are operably linked to other sequences such as control sequences which are capable of effecting the expression of said DNA sequences in a microorganism belonging to *Phaffia*. The term "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. The term "control sequence" is intended to include, at a minimum, components which are necessary for expression of the gene of interest, and may also include additional advantageous components. Generally the control sequences include promoters, terminators and, in some instances, enhancers, transactivators, or transcription factors. Constitutive promoters, such as the glyceraldehyde-3-dehydrogenase (GAP) gene

promoter derived from *P. rhodozyma* (WO 97/23,633), may be used to obtain constitutive expression. Inducible promoters can be also used in order to achieve an exactly-controlled expression. One of the examples for the inducible promoters is the promoter of genes encoding heat shock proteins or amylase gene, and the like.

5 Methods which are well known to those skilled in the art may be used to construct said expression vectors.

It is implied, although not explicitly stated, that expression vectors must be replicable in the host organisms either as episomes or as an integral part of a chromosomal DNA.

Generally, higher stability of the gene may be expected in the latter case. To integrate the  
10 expression vector into the chromosome of the host microorganism by a homologous recombination, a vector is prepared to contain at least a portion of the DNA fragment whose sequence is homologous to the host genomic DNA. For this purpose, rDNA gene fragment can be effectively used in a microorganism belonging to *Phaffia*. The rDNA is a kind of satellite DNAs which exist in multicopies on the genome. By using the rDNA fragment  
15 as a targeting DNA on the expression vector, the objective DNA to be expressed on said vector can be integrated into the host genome, and also can exist as multicopies. This may give the gene dosage effects which will contribute to the overexpression of the objective enzymes. In the Examples of the present invention, such rDNA fragment was conveniently used for this purpose. The invention is intended to include other forms of expression vectors  
20 which serve equivalent functions and which are, or subsequently become, known.

An isolated DNA sequence encoding  $\beta$ -carotene hydroxylase may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the nucleotide sequence encoding said  $\beta$ -carotene hydroxylase prior to its insertion into an expression vector may be desirable or necessary depending on the expression vector. The techniques for modifying nucleotide sequences utilizing cloning methods are well known in the  
25 art.

Recombinant DNA consisting of said gene of  $\beta$ -carotene hydroxylase which was cloned in the expression vector will be introduced into a host microorganism. Methods for the introduction of foreign DNA into fungal cells including a microorganism belonging to  
30 *Phaffia* are well known in the art. These include, e.g., transformation by the LiCl method, protoplast fusion, electroporation, particle-gun methods which comprises bombardment by particles coated with DNAs, and other methods known in the art. In the Examples of the present invention, the particle-gun method was applied as a transformation method for *P. rhodozyma*.

The recombinant organism thus obtained is capable of overexpressing the DNA sequence encoding  $\beta$ -carotene hydroxylase. Thus, the recombinant organism of the present invention is useful in the production process of xanthophyll carotenoids, especially zeaxanthin and  $\beta$ -cryptoxanthin.

- 5 A further aspect of the present invention is a biological process for producing zeaxanthin and  $\beta$ -cryptoxanthin which comprises cultivating the recombinant microorganism of *Phaffia* in the presence of substrate for producing carotenoids in an aqueous nutrient medium under aerobic conditions, and isolating the resulting carotenoids from the cells of said recombinant microorganism or from the cultured broth.
- 10 Carotenoids including the xanthophylls are normally produced by cultivating a strain of *Phaffia* in a medium which comprises suitable macro- and micronutrients for the cells, such as molasses, saccharose or glucose as a carbohydrate source for cell growth and also as a substrate for producing carotenoids, and nitrogen sources such as corn steep liquor, yeast extract, diammonium sulphate, ammonium phosphate, ammonium hydroxide or
- 15 urea, phosphorus sources such as ammonium phosphate and phosphoric acid and added micronutrients or mineral salts such as magnesium sulphate, zinc sulphate and biotin or desthiobiotin.

The preferable conditions for cultivation are in a pH range of from 4 to 8 and in a temperature range of from 15 to 26°C for 24 to 500 hours.

- 20 More preferable conditions for cultivation are a pH in the range of from 5 to 7 and in a temperature range of from 18 to 22°C for 48 to 350 hours.

In the cultivation, aeration and agitation usually give favorable results for the production of carotenoids.

- 25 Once carotenoids were produced by cultivating the recombinant strain of *Phaffia* using the methods of the present invention, the carotenoids can be isolated either from the medium, in the case they are secreted into the medium, or from the cells of the microorganism and, if necessary, separated from other carotenoids that may be present in case one specific carotenoid is desired, by methods known in the art.

- 30 Carotenoids produced in accordance with the present invention can be used in a process for the preparation of food or feed. A man skilled in the art is familiar with such processes. Such compound food or feed can further comprise additives or components generally used for such purpose and known in the state of the art.

The following Examples further illustrate the present invention, but these are not thereby limiting the scope of the invention.

The following materials and methods were employed in the Examples described below:

#### Strains

5 P. rhodozyma ATCC96594 (re-deposited under the accession No. ATCC 74438 on April 8, 1998 pursuant to the Budapest Treaty)

P. rhodozyma ATCC96815 (re-deposited under the accession No. ATCC 74486 on February 18, 1999 pursuant to the Budapest Treaty)

10 E. coli TOP10: F<sup>-</sup>, mcrA, delta(mrr-hsdRMS-mcrBC), phi80, delta(lacZ M15), delta(lacX74), recA1, deoR, araD139, (ara-leu)7697, galU, galK, rpsL (Str<sup>r</sup>), endA1, nupG (Invitrogen Corporation, Carlsbad, USA)

#### Vectors

pCR2.1-TOPO (Invitrogen Corporation, Carlsbad, USA)

pGEM-T (Promega Corporation, USA)

15 **Methods**

Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo (Ohtsu, Japan).

20 Polymerase chain reaction (PCR) was performed with the thermal cycler from Perkin Elmer model 2400. Each PCR condition is described in examples. PCR primers were purchased from a commercial supplier. Fluorescent DNA primers for DNA sequencing were purchased from Pharmacia. DNA sequencing was performed with the automated fluorescent DNA sequencer (ALFred, Pharmacia).

25 An authentic sample for zeaxanthin and  $\beta$ -carotene was purchased from EXTRASYN-THESE S.A. (Genay Cedex, France) and WAKO (Osaka, Japan), respectively.  $\beta$ -cryptoxanthin was obtained from Roche Vitamins AG (Basle, Switzerland).

**Example 1: Cloning of the  $\beta$ -carotene hydroxylase gene (*crtZ*) from genomic DNA of *Flavobacterium* sp. ATCC21588**

At first genomic DNA was prepared. Cells of *Flavobacterium* sp. R1534 WT (ATCC21588) (US 6,124,113) were inoculated into a 50 ml medium containing 1% glucose, 1% tryptone (Difco Laboratories), 1% yeast extract (Difco), 0.5% MgSO<sub>4</sub>, and 3% NaCl, and cultivated at 27°C under aerobic condition. Overnight culture (50 ml) was centrifuged at 10,000 g for 10 min. The pellet was washed briefly with 10 ml of lysis buffer (50 mM EDTA, 0.1M

NaCl, pH 7.5), resuspended in 4 ml of the same buffer supplemented with 10 mg of lysozyme and incubated at 37°C for 15 min. After addition of 0.3 ml of N-Lauroyl sarcosine (20%), incubation at 37°C was continued for another 15 min before extraction of DNA with phenol, phenol/chloroform and chloroform. The DNA was ethanol precipitated at 5 room temperature for 20 min in the presence of 0.3 M sodium acetate (pH 5.2), followed by centrifugation at 10,000 g for 15 min. The pellet was rinsed with 70% ethanol, dried and resuspended in 1 ml of TE (10 mM Tris, 1 mM EDTA, pH 8.0). Thus obtained genomic DNA was dialysed against H<sub>2</sub>O for 48 hours using collodium bags (Sartorius, Germany), ethanol precipitated in the presence of 0.3 M sodium acetate and resuspended 10 in H<sub>2</sub>O. Using the prepared genomic DNA as template, a complete coding sequence for the β-carotene hydroxylase gene without excessive flanking region was obtained by PCR amplification using a thermal cycler (Perkin elmer 2400, USA). The synthetic primers used were: PZ1 (SEQ ID NO:1)(having a *Sma* I site CCCGGG) and PZ2 (SEQ ID NO:2)(having a *Bam*H I site GGATCC).

15 The PCR was performed using an Advantage-HF PCR Kit (CLONTECH Laboratories, Inc., USA). The PCR mixture was prepared according to the user's manual described by the manufacturer. The initial template denaturation step consisted of 1 min at 94°C. An amplification cycle of 30 seconds at 94°C and 4 min at 68°C was repeated for 25 times. After additional 3 min reaction at 68°C, the reaction mixture was kept at 15°C. By this 20 reaction, DNA fragment containing a complete ORF of the β-carotene hydroxylase gene (522 bp) was amplified. This amplified β-carotene hydroxylase gene was ligated with a pCR2.1-TOPO vector and introduced into *E.coli* TOP 10 cells by using a TOPO TA Cloning kit (Invitrogen Corporation, USA) according to an instruction manual prepared by the manufacturer. Several clones were selected for sequence analysis. The sequence of 25 the cloned β-carotene hydroxylase gene of each candidate clone was examined. One of the DNA clones that showed completely the same sequence as the β-carotene hydroxylase sequence of *Flavobacterium* sp. ATCC21588 (GenBank accession No. U62808) was named as pTOPO-PZ#23N. A 522 bp *Sma* I / *Bam*H I fragment cut out from pTOPO-PZ#23N was used as a *crtZ* gene cassette of *Flavobacterium* sp.

30 **Example 2: Cloning of the β-carotene hydroxylase gene (*crtZ*) from genomic DNA of *E. herbicola* ATCC39368**

Cells of *E. herbicola* (*Escherichia vulneris*) ATCC39368 were inoculated into a 10 ml medium containing 1% tryptone (Difco Laboratories), 0.5% yeast extract (Difco), 0.5% NaCl, and cultivated at 26°C under aerobic conditions. Overnight culture (10 ml) was

centrifuged at 10,000 g for 10 min. The pellet was used for the genomic DNA preparation using a QIAGEN Blood & Cell Culture DNA Midi Kit (QIAGEN, Germany) according to a protocol prepared by the manufacturer. Using the prepared genomic DNA as template, a complete coding sequence for the  $\beta$ -carotene hydroxylase gene without excessive flanking 5 region was obtained by PCR amplification using a thermal cycler (Perkin elmer 2400, USA). The two synthetic primers used were EZ1 (SEQ ID NO:3)(having an *Sma* I site CCCGGG) and EZ2 (SEQ ID NO:4)(having an *Sal* I site GTCGAC). The PCR was performed using an Advantage-HF PCR Kit (CLONTECH Laboratories, Inc., USA). The PCR mixture was prepared according to the user's manual described by 10 the manufacturer. The initial template denaturation step consisted of 1 min at 94°C. An amplification cycle of 30 seconds at 94°C and 4 min at 68°C was repeated for 25 times. After additional 3 min reaction at 68°C, the reaction mixture was kept at 15°C. By this 15 reaction, DNA fragment containing a complete ORF of the  $\beta$ -carotene hydroxylase gene (543 bp) was amplified. This amplified  $\beta$ -carotene hydroxylase gene was ligated with a pCR2.1-TOPO vector and introduced into *E.coli* TOP 10 cells by using a TOPO TA Cloning kit (Invitrogen Corporation, USA) according to an instruction manual prepared by the manufacturer. Several clones were selected for sequence analysis. The sequence of the cloned  $\beta$ -carotene hydroxylase gene of each candidate clone was examined. One of the 20 DNA clones that showed completely the same sequence as the  $\beta$ -carotene hydroxylase sequence of *E. herbicola* ATCC39368 (GenBank accession No. M87280) was named as pTOPO-EZ#2. A 543 bp *Sma* I / *Sal* I fragment cut out from pTOPO-EZ#2 was used as a *crtZ* gene cassette of *E. herbicola*.

#### Example 3: Preparation of components of the expression vector

The components of the expression vector, a G418 resistant gene, promoter and terminator 25 region of glyceraldehyde-3-phosphate dehydrogenase gene (hereinafter referred to as GAP) of *P. rhodozyma*, and rDNA fragment of *P. rhodozyma* were prepared. A G418 resistant gene cassette was prepared as follows. A *Sac* I- linker was ligated into the unique *Hind* III site of the vector, pUC-G418 (US Patent No. 6,365,386 B1), which is harboring the G418 resistant gene cassette, and resulted vector was named as pG418Sa512. 30 A 1.7 kb *Kpn* I / *Sac* I fragment cut out from pG418Sa512 was used as a G418 resistant gene cassette.

Each of the promoter and the terminator of GAP gene and the rDNA fragment was obtained by PCR using the genomic DNA of *P. rhodozyma* ATCC 96594 as template. To obtain the genomic DNA, a QIAGEN Blood & Cell Culture DNA Midi Kit (QIAGEN,

Germany) was used with the cells of *P. rhodozyma* ATCC 96594 obtained by overnight culture in YPD (Difco Laboratories) medium.

Using the prepared genomic DNA as a template, PCR was performed using an Advantage-HF PCR Kit (CLONTECH Laboratories, Inc., USA) and a thermal cycler (Perkin elmer

5 2400, USA).

The two synthetic primers used to amplify the promoter sequence of GAP were GAP#1 (SEQ ID NO:5)(having a *Not* I site GCGGCCGC) and GAP#5 (SEQ ID NO:6)(having a *Sma* I site CCCGGGGG).

The initial template denaturation step consisted of 5 min at 94°C. An amplification cycle 10 of 30 seconds at 94°C, 30 seconds at 55°C, and 1 min at 72°C was repeated for 25 times.

After additional 10 min reaction at 72°C, the reaction mixture was kept at 4°C. By this reaction, DNA fragment containing the GAP promoter (398 bp) was amplified. This amplified GAP promoter was ligated with a pCR2.1-TOPO vector and introduced into *E.coli* TOP 10 cells by using a TOPO TA Cloning kit (Invitrogen Corporation, USA).

15 Several clones were selected for sequence analysis. The sequence of the cloned GAP promoter of each candidate clone was examined. One of the DNA clones that showed completely the same sequence as the GAP promoter of *P. rhodozyma* (GenBank accession No. Y08366) was named as pTOPO-pGAP2#1. A 398 bp *Not* I / *Sma* I fragment cut out from pTOPO-pGAP2#1 was used as the GAP promoter cassette.

20 The synthetic primers used to amplify the terminator sequence of GAP were GAP#33 (SEQ ID NO:7)(having a *Bam*H I-*Sal* I site GGATCCGTCGAC) and GAP#4 (SEQ ID NO:8) (having a *Kpn* I siteGGTACCC).

The PCR conditions were the same as those for the GAP promoter described above. By this reaction, DNA fragment containing the GAP terminator (302 bp) was amplified. This 25 amplified GAP terminator was ligated with a pCR2.1-TOPO vector and introduced into *E.coli* TOP 10 cells by using the TOPO TA Cloning kit. Several clones were selected for sequence analysis. The sequence of the cloned GAP terminator of each candidate clone was examined. One of the DNA clones that showed completely the same sequence as the GAP terminator of *P. rhodozyma* (GenBank accession No. Y08366) was named as pTOPO-tGAP#1. A 302 bp *Bam*H I / *Kpn* I fragment or a 296 bp *Sal* I / *Kpn* I fragment which was cut out from pTOPO-tGAP#1 was used as the GAP terminator cassette.

The two synthetic primers used to amplify the rDNA fragment were R#1 (SEQ ID NO:9) (having a *Sac* I site GAGCTC) and R#2 (SEQ ID NO:10)(having a *Not* I-*Sac* I site GCGGCCCGGAGCT).

35 The PCR conditions were the same as those for the GAP promoter described above. By this reaction, DNA fragment containing the rDNA (3126 bp) was amplified. This ampli-

fied the rDNA was ligated with a pCR2.1-TOPO vector and introduced into *E.coli* TOP 10 cells by using the TOPO TA Cloning kit. Several clones were selected for sequence analysis. The sequence of the cloned the rDNA of each candidate clone was examined. One of the DNA clones that showed completely the same sequence as the rDNA of *P. rhodozyma* (GenBank accession No. D31656, AF139632) was named as pTOPO-rDNA#1. A 1960 bp *Sac* II / *Not* I fragment cut out from pTOPO-rDNA#1 was used as the rDNA cassette.

**Example 4: Construction of the expression vector carrying the  $\beta$ -carotene hydroxylase gene (*crtZ*) obtained from *Flavobacterium* sp. or *E. herbicola***

To construct the expression vector carrying the *crtZ* of *Flavobacterium* sp., the rDNA cassette and the GAP promoter cassette (obtained in Example 3), the *crtZ* gene cassette of *Flavobacterium* sp. (obtained in Example 1), and the *Bam* H I / *Kpn* I fragment of the GAP terminator cassette and the G418 resistant gene cassette (obtained in Example 3) were aligned by ligation in this order using a pGEM-T as a backbone. The resulting expression vector was named as pRPZG-ptGAP2.

To construct the expression vector carrying the *crtZ* of *E. herbicola*, the rDNA cassette and the GAP promoter cassette (obtained in Example 3), the *crtZ* gene cassette of *E. herbicola* (obtained in Example 2), and *Sal* I / *Kpn* I fragment of the GAP terminator cassette and the G418 resistant gene cassette (obtained in Example 3) were aligned by ligation in this order using a pGEM-T as a backbone. Resulted expression vector was named as pREZG-ptGAP2.

**Example 5: Introduction of the expression vector carrying the  $\beta$ -carotene hydroxylase gene (*crtZ*) of *Flavobacterium* sp. or *E. herbicola* into *P. rhodozyma***

Each of the expression vectors obtained in the Example 4, pRPZG-ptGAP2 and pREZG-ptGAP2, carrying the *crtZ* gene of *Flavobacterium* sp. and *E. herbicola*, respectively, was introduced into *P. rhodozyma* ATCC 96815, a mutant strain producing  $\beta$ -carotene predominantly by using the particle-gun method as described in EP 1,158,051. YPD-agar medium containing 0.75 M D-sorbitol and D-mannitol, and 0.1 mg/ml of geneticin was used for direct selection of the recombinant strains.

As a result, hundreds of transformants which were resistant to 0.1 mg/ml geneticin were obtained by each of pRPZG-ptGAP2 and pREZG-ptGAP2, and 35 each transformants were selected randomly for further characterization.

**Example 6: Characterization of the *crtZ*-recombinant strains of *P. rhodozyma***

The *crtZ*-recombinant strains of *P. rhodozyma* ATCC 96815 thus obtained were cultivated in 50 ml production medium in 500 ml Erlenmeyer flask with shaking at 20°C for 7 days after inoculation of seed culture prepared in 7 ml seed medium in test tube (21 mm in diameter) with shaking at 20°C for 3 days. Appropriate volume of the culture broth was withdrawn and used for analysis of the cell growth and the productivity of carotenoids. Medium composition was as follows:

**Seed medium:** Glucose 30.0 g/l, NH<sub>4</sub>Cl 4.83 g/l, KH<sub>2</sub>PO<sub>4</sub> 1.0 g/l, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.88 g/l, NaCl 0.06 g/l, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.2 g/l, KH phtalate 20.0 g/l, FeSO<sub>4</sub>·7H<sub>2</sub>O 28 mg/l, Trace element solution 0.3 ml, Vitamin stock solution 1.5 ml, (pH was adjusted at 5.4–5.6).

**Trace element solution:** 4N- H<sub>2</sub>SO<sub>4</sub> 100 ml/l, citric acid-H<sub>2</sub>O 50.0 g/l, ZnSO<sub>4</sub>·7H<sub>2</sub>O 16.7 g/l, CuSO<sub>4</sub>·5H<sub>2</sub>O 2.5 g/l, MnSO<sub>4</sub>·4,5H<sub>2</sub>O 2.0 g/l, H<sub>3</sub>BO<sub>3</sub> 2.0 g/l, Na<sub>2</sub>MoO<sub>4</sub> 2.0 g/l, KI 0.5 g/l.

**Vitamin stock solution for seed medium:** 4N- H<sub>2</sub>SO<sub>4</sub> 17.5 ml/l, *myo*-Inositol 40.0 g/l, Nicotinic acid 2.0 g/l, Ca-D-pantothenate 2.0 g/l, Vitamin B<sub>1</sub> (thiamin HCl) 2.0 g/l, *p*-Aminobenzoic acid 1.2 g/l, Vitamin B<sub>6</sub> (pyridoxine HCl) 0.2 g/l, Biotin stock solution 8.0 ml.

**Biotin stock solution** was prepared by the addition of 4N- H<sub>2</sub>SO<sub>4</sub> to 50 ml of ethanol to a total of 100 ml. Then, 400 mg of D-biotin were added.

**Production medium:** Glucose 22.0 g/l, KH<sub>2</sub>PO<sub>4</sub> 14.25 g/l, MgSO<sub>4</sub>·7H<sub>2</sub>O 2.1 g/l, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.865 g/l, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 3.7 g/l, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.28 g/l, Trace element solution 4.2 ml, Vitamin stock solution 9.35 ml, (pH was adjusted at 5.5).

**Vitamin stock solution for production medium:** 4N- H<sub>2</sub>SO<sub>4</sub> 17.5 ml/l, Nicotinic acid 2.0 g/l, Ca-D-pantothenate 3.0 g/l, Vitamin B<sub>1</sub> (thiamin HCl) 2.0 g/l, *p*-Aminobenzoic acid 1.2 g/l, Vitamin B<sub>6</sub> (pyridoxine HCl) 0.2 g/l, Biotin stock solution 30.0 ml

A portion of the seed culture broth (2.5 ml) was transferred to 47.5 ml of the production medium in 500 ml Erlenmeyer flask. Then the cultivation was started at 20°C with rotary shaking at 200 rpm. At the second day of the fermentation, 5 ml of 50 % glucose solution was added to the medium and then the fermentation was continued. At the fourth day of the fermentation, 2 ml of cultured broth was withdrawn and then 5 ml of 50 % glucose solution was added again to the medium, and cultivation was continued for additional 3 days. At the seventh day, an aliquot of the culture was withdrawn and used for analysis of the carotenoids production and the cell growth.

For analysis of the cell growth, optical density at 660 nm was measured by using UV-1200 photometer (Shimadzu Corp., Kyoto, Japan).

For analysis of the content of  $\beta$ -carotene,  $\beta$ -crypto-xanthin and zeaxanthin, the withdrawn broth was mixed with a solvent mixture (ethyl alcohol, hexane and ethyl acetate) and carotenoids were extracted from the broth and the cells of *P. rhodozyma* by vigorous shaking with glass beads. After extraction, disrupted cells and glass beads were removed by 5 centrifugation and the resultant supernatant was analyzed by HPLC for the carotenoids content. The HPLC conditions used is as follows: HPLC column: Chrompack Lichrosorb si-60 (4.6 mm, 250 mm); Temperature: room temperature; Eluent: acetone / hexane (18/82) add 1 ml / l of water to eluent; Injection volume: 10  $\mu$ l, Flow rate: 2.0 ml / minute; Detection: UV at 450 nm.

10 As a result, it was found that all recombinant strains (35 each transformants for two kinds of *crtZ*-expression vectors, pRPZG-ptGAP2 and pREZG-ptGAP2) produced zeaxanthin and  $\beta$ -crypto-xanthin without accumulation of astaxanthin.

15 Fermentation profile of the representative 3 each recombinant strains selected from the transformants expressing *crtZ* gene of *Flavobacterium* sp. or *crtZ* gene of *E. herbicola*, respectively, is shown in the Table together with that of their host strain (*P. rhodozyma* ATCC 96815).

Table: Carotenogenesis and growth profile of the recombinant strains in shake flask culture

Strain	Origin of <i>crtZ</i>	CARO (mg/l)	CRYPTO (mg/l)	ZEA (mg/l)	ASTA (mg/l)	OD at 660 nm
PZ-7	<i>Flavobacterium</i> sp.	4.7	3.5	5.7	0.0	46.3
PZ-24	<i>Flavobacterium</i> sp.	7.1	2.7	2.7	0.0	48.3
PZ-25	<i>Flavobacterium</i> sp.	6.3	2.1	2.7	0.0	48.3
EZ-7	<i>E. herbicola</i>	3.3	2.7	2.2	0.0	49.0
EZ-13	<i>E. herbicola</i>	3.3	2.8	2.3	0.0	44.7
EZ-14	<i>E. herbicola</i>	3.6	3.1	2.4	0.0	45.9
ATCC 96815	-	10.2	0.0	0.0	0.0	51.9

CARO:  $\beta$ -carotene; CRYPTO:  $\beta$ -crypto-xanthin; ZEA: Zeaxanthin; ASTA: Astaxanthin

20 As shown in the Table, conversion from  $\beta$ -carotene into  $\beta$ -crypto-xanthin and zeaxanthin was exemplified in all the *Phaffia* recombinant strains expressing *crtZ* gene of *Flavobacterium* sp. or *crtZ* gene of *E. herbicola*.

## Claims

1. A process for producing zeaxanthin and  $\beta$ -cryptoxanthin which comprises cultivating a recombinant microorganism which is expressing a  $\beta$ -carotene hydroxylase gene and belonging to the genus *Xanthophyllomyces* (*Phaffia*) in an aqueous nutrient medium under aerobic conditions, and isolating the resulted carotenoids from the cells of said recombinant microorganism or from the cultured broth.
2. The process according to claim 1, wherein the recombinant microorganism is derived from *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) ATCC96815, or a mutant thereof.
- 10 3. The process according to claim 1 or 2, wherein the  $\beta$ -carotene hydroxylase gene is originated from a microorganism which is selected from the group consisting of microorganisms of the genera *Flavobacterium*, *Erwinia*, *Agrobacterium*, *Alcaligenes*, and *Paracoccus*, which are having the  $\beta$ -carotene hydroxylase gene.
4. The process according to claim 1 or 2, wherein the  $\beta$ -carotene hydroxylase gene is originated from a microorganism which is selected from the group consisting of *Flavobacterium* sp. R1534 WT (ATCC21588), *Erwinia uredovora* ATCC19321, *Erwinia herbicola* ATCC39368, *Agrobacterium aurantiacum*, *Alcaligenes* PC-1, *Paracoccus marcusii* MH1, and a gram-negative bacteria E-396 (FERM BP-4283) which are having the  $\beta$ -carotene hydroxylase gene.
- 20 5. The process according to claim 1 or 2, wherein the  $\beta$ -carotene hydroxylase gene is originated from *Flavobacterium* sp. R1534 WT (ATCC21588) or the DNA sequence of the  $\beta$ -carotene hydroxylase gene is substantially homologous thereto.
6. The process according to any one of claims 1 to 5, wherein the  $\beta$ -carotene hydroxylase gene is expressed in the recombinant microorganism using the control sequences.
- 25 7. The process according to any one of claims 1 to 6, wherein the cultivation is carried out at pH range from 4 to 8 and at a temperature range from 15 to 26°C for 24 to 500 hours.
8. The process according to claim 7, wherein the cultivation is carried out at pH range from 5 to 7 and at a temperature range from 18 to 22°C for 48 to 350 hours.

sequence listing-21415.ST25  
SEQUENCE LISTING

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<120> Production of zeaxanthin by Phaffia

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<170> PatentIn version 3.1

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28

## INTERNATIONAL SEARCH REPORT

PCT/EP 3/10574

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12P23/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, EMBASE, PAJ

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Y	EP 0 747 483 A (HOFFMANN LA ROCHE) 11 December 1996 (1996-12-11) claims 1,13,25,27 example 7 page 4, line 22,23 page 5, line 2-13 ----	1-8
Y	WO 94 06918 A (GIST BROCADES NV) 31 March 1994 (1994-03-31) page 8, line 4-6 page 9, line 36 -page 10, line 6 page 10, line 2-6,30-36 page 11, line 1,2 page 12 -page 17 page 21, line 7,8 ---- -/-	1-8

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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Date of the actual completion of the international search

30 December 2003

Date of mailing of the international search report

28/01/2004

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## INTERNATIONAL SEARCH REPORT

PCT/ISA/3/10574

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A	EP 0 933 427 A (BIO ORIENTED TECHNOLOGY RESEAR ;DIRECTOR GENERAL OF NATIONAL I (JP) 4 August 1999 (1999-08-04) page 6, paragraph 44 page 9, paragraph 87 ----	1-8
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A	US 6 291 204 B1 (PASAMONTES LUIS ET AL) 18 September 2001 (2001-09-18) column 5, line 45,46 claims 1,2 -----	1-8

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